PREPARATION OF THERMO-RESPONSIVE POLYMER GELS IMMOBILIZING CORE-SHELL TYPE BIOCONJUGAYES

Atsushi Harada, Kunio Johnin, and Kenji Kono
Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

Introduction

Immobilization of enzymes is interesting research topic from both fundamental and applied aspects, and various kinds of immobilization methods including chemical and physical procedures have been actively investigated, e.g. the chemical conjugation of enzyme with water-soluble polymers and the physical entrapment into polymer gel matrix (1-5). The advantages of enzyme-immobilization were improvement of storage and operational stabilities. In such studies, the activity of enzyme often decreased after chemical or physical immobilization, although the stabilities were improved. The decrease in enzymatic activity might be induced by the change in environment around enzyme molecule and/or the change in conformation of enzyme molecule through chemical and physical enzyme-immobilization. If the enzyme-immobilization method in consideration of the change in environment and conformation is used, it was expected that it was possible to prevent the decrease in enzyme activity with immobilization. For the chemical conjugation of enzymes and water-soluble polymers concerning limited combinations, which is one of chemical immobilization method, it has been reported that the storage stability of enzyme may improve without a decrease in enzymatic activity. As one of such bioconjugate system, we have recently succeeded the preparation of core-shell type bioconjugates from the mixture of bovine pancreas trypsin and poly(ethylene glycol)-block-poly(α,β-aspartic acid) (PEG-PAA) through the crosslinking between trysin and block ionomer by glutaraldehyde (6). The enzymatic activity of trypsin in the core of core-shell type bioconjugates was higher than that of native trypsin, and both the storage stability and operational stability was drastically improved. When core-shell type bioconjugates were physically entrapped in polymer gel matrix by entangled effect, it was expected that the function of enzyme might be maintained even after the entrapment. Because the conformation of trypsin molecules in the bioconjugates was fixed by the glutaraldehyde crosslinking, and the surface of the bioconjugates was surrounded by PEG shell layer. Enzyme molecules in core-shell type bioconjugates were surrounded by polymer chains. Enzyme molecules might not directly interact with the polymer gel matrix, when bioconjugates entrapped in the polymer gels. Consequently, the functions of enzymes might be maintained even after the entrapment in the polymer gels.

In this study, core-shell type bioconjugates entrapping trypsin molecules in the core was physically entrapped in temperature-responsive polymer gel, poly(N-isopropylacrylamide) (PNIPAAm) gel (Figure 1). The effect of entrapment to phase transition temperature of polymer gels and enzymatic activity of biocojugates

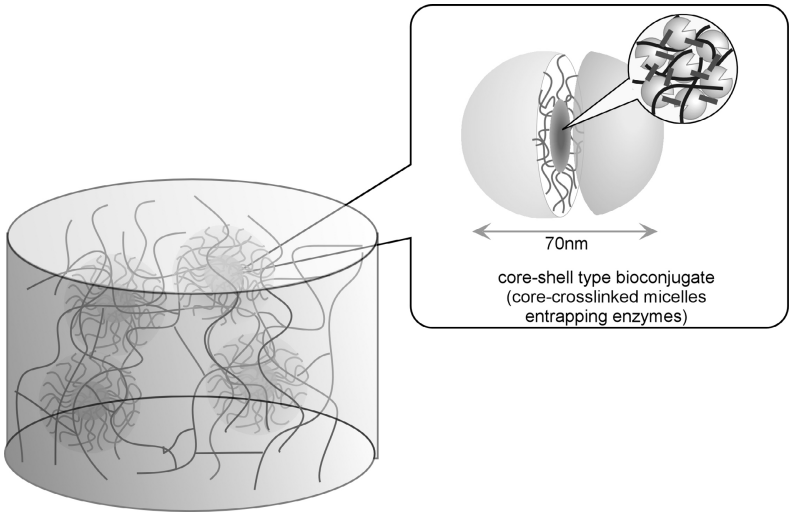

Figure 1. Schematic image of polymer gels entrapping core-shell type bioconjugates

were evaluated. It was confirmed that the functions of PNIPAAm gels and core-shell type bioconjugates were maintained even after the incorporation.

Experimental Part

Poly(ethylene glycol)-poly(α,β-aspartic acid) block copolymer [PEG-PAA; 12000 g/mol of PEG Mw; 28 polymerization degree of PAA] was synthesized as already described (7). N-isopropylacrylamide and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were purchased from Wako Pure Chemical Co., Ltd and purified by recrystallization. N, N'-methylenebisacrylamide (MBA), N, N, N', N'-tetramethylethylenediamine(TEMED), potassium persulfate (KPS), and 70% glutaraldehyde aqueous solution were purchased from Wako Pure Chemical Co., Ltd., and bovine pancreas trypsin and L-lysine-*p*-nitroanilide were purchased from Sigma, and used without further purification.

Core-shell type bioconjugates, core-crosslinked PIC micelles entrapping enzyme molecules in the core, were prepared as follows: Given amounts of trypsin and poly(ethylene glycol)-poly(α,β-aspartic acid) block copolymer [PEG-PAA; 12000 g/mol of PEG Mw; 28 of polymerization degree in PAA segment] were separately dissolved in sodium phosphate buffer (10 mM; pH 7.4) at 4°C. The solutions were mixed at the optimum mixing ratio for PIC micelle formation, i.e., the ratio of the number of Asp residues in PEG-PAA against the total number of Lys and Arg residues in trypsin to be 0.75. The mixed solution was stored at 4 °C for 30 min, and then, was added 70% glutaraldehyde solutions to prepare crosslinked micelles. The added amount of glutaraldehyde was shown as the glutaraldehyde crosslinking ratios (GR), which was defined as the number of aldehyde groups of glutaraldehyde against the number of Lys residues in trypsin. The crosslinked PIC micelle solution was then kept at 4 ^{o}C for 30 min. In order to remove excess glutaraldehyde, the crosslinked PIC micelle solution was dialyzed against the buffer. The removal of free glutaraldehyde was confirmed from the absence of aldehyde species in dialysate by colorimetric assay using 3-methyl-2-benzothiazolinone hydrazone. Then, $NaBH_3CN$ was added to the solution to convert Schiff base linkages to alkylamine linkages by the reductive amination. The reductive amination was also confirmed by colorimetric assay using 3-methyl-2-benzothiazolinone hydrazone. The average size of the obtained core-shell type bioconjugates were confirmed by dynamic light scattering measurements.

PNIPAAm gels containing core-shell type bioconjugates were prepared by redox polymerization in 96-well plates using TEMED and KPS as radical initiator, in presence of varying amounts of core-shell type bioconjugates and MBA as a cross-linking regent. The feed compositions for each gel are summarized in Table 1. After 20 hours at 4°C, each gel was immersed in distilled water for 2 days in order to remove unreacted monomers. In this washing process, the amount of un-entrapped bioconjugates was determined by TNBS analysis, and then, the amount of entrapped biocojugates was calculated. Water content of the gels was calculated from the following equation:

$$(\text{water content}) = (W_T - W_D) / W_D$$

where W_T and W_D were weights of swelling gels at T ^{o}C and freeze-drying gels, respectively. Also, enzymatic activity of bioconjugates was evaluated by colorimetric assay using L-lysine-*p*-nitroanilide as a substrate.

Table 1. Feed compositions of PNIPAAm gels entrapping core-shell type bioconjugates

code	NIPAAm [mmol]	MBA [μmol]	cross-linking density [mol%]	bioconjugate [μg]	KPS [mg]	TEMED [μL]
2B15	1.53	32	2	150	7.2	14
5B8	1.43	80	5	80	7.2	14
5B15	1.43	80	5	150	7.2	14
5B27	1.43	80	5	270	7.2	14
5B39	1.43	80	5	390	7.2	14
7B15	1.37	110	7	150	7.2	14
11B15	1.27	160	11	150	7.2	14

* The volume of reaction solutions was fixed to be 3 mL for all compositions.

Results and Discussion

The amounts of core-shell type bioconjugates entrapped in the PNIPAAm gels were independent on the cross-linking density and loading amount of bioconjugates, and the entrapping efficiency for all gels was determined to be ca. 70 %. When the gels prepared under the conditions summarized in Table 1, the finally obtained gels had the volume of ca. 70 % against the volume of reaction mixtures. When the gelling reaction progressed to the state in which core-shell type bioconjugates could not move, the bioconjugates which existed in gelling space might be entrapped in PNIPAAm gels. Also, after washing several times, there was no detection of the release of bioconjugates from the gels. The bioconjugates had average diameter of 70 nm, and they might not be able to diffuse in the gel matrix due to the size effect.

Figure 2 show the change in water content of the PNIPAAm gels entrapping varying amounts of bioconjugates with temperature, in which the PNIPAAm gels prepared at the cross-linking density of 5 mol%. It was obvious that the water contents of the gels drastically changed at 32 °C, and the entrapment of bioconjugates in the PNIPAAm gels was no influence to the phase transition temperature. There was the difference in the water contents below phase transition temperature. The water contents of the gels increased with an increase in the loading amounts of bioconjugates. This might be due to the effect of hydration of core-shell type bioconjugates, which had PEG brush layer as a shell.

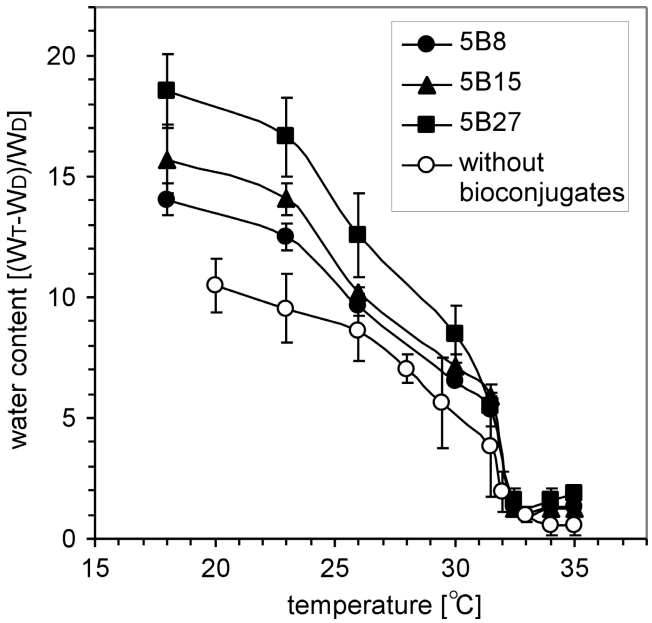

Figure 2. Change in water contents of the PNIPAAm gels entrapping varying amounts of core-shell type bioconjugates with temperature. (n=3)

The enzymatic activity of core-shell type biocojugates in the gels was evaluated by colorimetric assay using L-lysine-*p*-nitroanilide as a substrate. Even in the PNIPAAm gel matrix, trypsin in core-shell type bioconjugates showed its amidase activity, and *p*-nitroaniline was released. The reaction rate was determined

from the released amount of *p*-nitroaniline. Figure 3 shows Arrhenius plots of the reaction rate of core-shell type bioconjugate in solution and in PNIPAAm gels. Although the core-shell type bioconjugates in solution showed one linear plots, bioconjugates in the gels showed two linear regions bounded at phase transition temperature shown in Figure 2. The absolute value of reaction rate for enzymes in the shrinking gels was lower than those in the swelling gels and in the solution. Interestingly, the slope of core-shell type bioconjugates in solution and those in the gels at both below and above phase transition temperature were almost same, suggesting that enzymes maintained even in the PNIPAAm gel at above phase transition temperature. This result indicates that the entrapment of bioconjugates in the PNIPAAm gels was no influence to the enzymatic functions of bioconjugates.

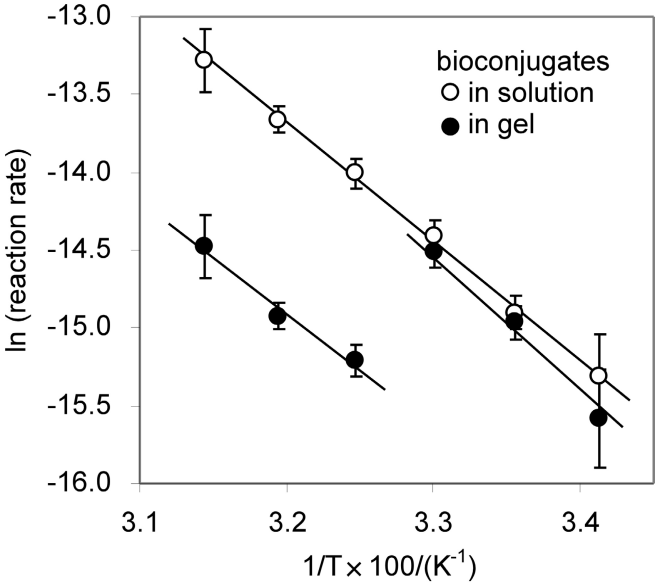

Figure 3. Arrhenius plots of reaction rate of core-shell type bioconjugates in solution and in PNIPAAm gels (n=3)

It was confirmed that the properties of PNIPAAm gels and core-shell type bioconjugates under equilibrium conditions were independent, and did not influence each other.

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